PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 15/00	A2	(11) International Publication Number: WO 99/03986
		(43) International Publication Date: 28 January 1999 (28.01.99)

(21) International Application Number: PCT/US98/14911

(22) International Filing Date: 17 July 1998 (17.07.98)

(30) Priority Data:
08/896,323 17 July 1997 (17.07.97) US

(71) Applicant: KOSAN BIOSCIENCES, INC. [US/US]; 1450 Rollins Road, Burlingame, CA 94010 (US).

(72) Inventors: KHOSLA, Chaitan; 132 Peter Courts Circle, Stanford, CT 94305 (US). PIEPER, Rembert; 957 Menlo Avenue, Menlo Park, CA 94025 (US). LUO, Guanglin; Apartment #3, 80 Preston Street, Providence, RI 02906 (US). CANE, David, E.; 35 Laural Avenue, Providence, RI 02906 (US).

(74) Agents: MURASHIGE, Kate, H. et al.; Morrison & Foerster LLP, 2000 Pennsylvania Avenue, N.W., Washington, DC 20006–1888 (US).

(81) Designated States: AL, AM, AU, AZ, BA, BB, BG, BR, CA, CN, CU, CZ, EE, FI, GE, HU, IL, IS, JP, KG, KP, KR, LC, LK, LR, LT, LV, MD, MG, MK, MN, MX, NO, NZ, PL, RO, SG, SI, SK, TR, TT, UA, UZ, VN, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

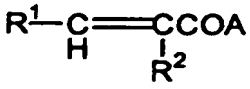
Published
Without international search report and to be republished upon receipt of that report.

(54) Title: METHOD TO PRODUCE NOVEL POLYKETIDES

(57) Abstract

Modified PKS gene clusters which produce novel polyketides in an efficient system in a host cell or in a cell free extract are described. The novel polyketides result from the incorporation of diketides of formula (1) or (2), wherein A is a molety that activates the diketide, and at least one of R^1 and R^2 is a substituent other than that natively occuring in the diketide normally processed by the modified PKS cluster. The polyketides may also be glycosylated to provide antibiotics.

(1)



(2)

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	**ES**	Spain	**LS**	Lesotho	**SI**	Slovenia
AM	Armenia	**FI**	Finland	**LT**	Lithuania	**SK**	Slovakia
AT	Austria	**FR**	France	**LU**	Luxembourg	**SN**	Senegal
AU	Australia	**GA**	Gabon	**LV**	Latvia	**SZ**	Swaziland
AZ	Azerbaijan	**GB**	United Kingdom	**MC**	Monaco	**TD**	Chad
BA	Bosnia and Herzegovina	**GE**	Georgia	**MD**	Republic of Moldova	**TG**	Togo
BB	Barbados	**GH**	Ghana	**MG**	Madagascar	**TJ**	Tajikistan
BE	Belgium	**GN**	Guinea	**MK**	The former Yugoslav Republic of Macedonia	**TM**	Turkmenistan
BF	Burkina Faso	**GR**	Greece			**TR**	Turkey
BG	Bulgaria	**HU**	Hungary	**ML**	Mali	**TT**	Trinidad and Tobago
BJ	Benin	**IE**	Ireland	**MN**	Mongolia	**UA**	Ukraine
BR	Brazil	**IL**	Israel	**MR**	Mauritania	**UG**	Uganda
BY	Belarus	**IS**	Iceland	**MW**	Malawi	**US**	United States of America
CA	Canada	**IT**	Italy	**MX**	Mexico	**UZ**	Uzbekistan
CF	Central African Republic	**JP**	Japan	**NE**	Niger	**VN**	Viet Nam
CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

METHOD TO PRODUCE NOVEL POLYKETIDES

Statement of Rights to Inventions Made under Federally Sponsored Research

This invention was made with U.S. government support from the National Institutes of Health (GM22172 and CA66736-01). The government has certain rights in this invention.

Technical Field

The invention relates to methods to synthesize polyketides which are novel using modified modular polyketides synthases (PKS) which cannot utilize a natural first module starter unit.

Background Art

Modular polyketide syntheses are typified by the organization of deoxyerythronolide B synthase (DEBS) which produces β-deoxyerythronolide B (6-dEB) the parent macrolactone of the broad spectrum antibiotic erythromycin. DEBS consists of three large polypeptides each containing about 10 distinctive active sites. Fig. 1 shows, diagrammatically, the nature of the three DEBS modules encoded by the three genes *eryAI*, *eryAII* and *eryAIII*.

Various strategies have been suggested for genetic manipulation of PKS to produce novel polyketides. New polyketides have been generated through module deletion (Kao, C.M. *et al.*, *J. Am. Chem. Soc.* (1995) 117:9105-9106; Kao, C.M. et al, *J. Am. Chem. Soc.* (1996) 118:9184-9185). Also reported to provide novel polyketides are loss of function mutagenesis within reductive domains (Donadio, S. *et al.*, *Science* (1991) 252:675-679; Donadio, S. et al, *Proc. Natl. Acad. Sci. USA* (1993) 90:7119-7123; Bedford, D. *et al.*, *Chem. Biol.* (1996) 3:827-831) and replacement of acyl transferase domains to alter starter or extender unit specificity (Oliynyk, M *et al.*, *Chem. Biol.* (1996) 3:833-839; Kuhstoss, S. *et al.*, *Gene* (1996) 183:231-236), as well as gain of function mutagenesis to introduce new catalytic activities within existing modules (McDaniel, R. *et al.*, *J. Am. Chem. Soc.* (1997) in press). In some of these reports, downstream enzymes in the polyketide pathway have been shown to process

non-natural intermediates. However, these methods for providing novel polyketides suffer from the disadvantages of requiring investment in cloning and DNA sequencing, the systems used being limited to producer organisms for which gene replacement techniques have been developed, primer and extender units that can only be derived from metabolically accessible CoA thioesters, and the fact that only limited auxiliary catalytic functions can be employed.

The DEBS system in particular has been shown to accept non-natural primer units such as acetyl and butyryl-CoA (Wiesmann, KEH et al, *Chem. Biol.* (1995) 2:583-589; Pieper, R. et al, *J. Am. Chem. Soc.* (1995) 117:11373-11374) as well as N-acetylcysteamine (NAC) thioesters of their corresponding diketides (Pieper, R. *et al.*, *Nature* (1995) 378:263-266). However, it has become clear that even though such unnatural substrates can be utilized, competition from the natural starter unit has drastically lowered yield. Even if starter units are not supplied artificially, they can be inherently generated from decarboxylation of the methylmalonyl extender units employed by the DEBS system (Pieper, R. *et al.*, *Biochemistry* (1996) 35:2054-2060; Pieper, R. *et al.*, *Biochemistry* (1997) 36:1846-1851).

Accordingly, it would be advantageous to provide a mutant form of the modular polyketide synthesis system which cannot employ the natural starter unit. Such systems can be induced to make novel polyketides by supplying, instead, a suitable diketide as an NAC thioester or other suitable thioester. Mutations have been made in the past to eliminate the competition from natural materials (Daum, S.J. *et al.*, *Ann. Rev. Microbiol.* (1979) 33:241-265). Novel avermectin derivatives have been synthesized using a randomly generated mutant strain of the avermectin producing organism (Dutton, C.J. *et al.*, *Tetrahedron Letters* (1994) 35:327-330; Dutton, C.J. *et al.*, *J. Antibiot.* (1991) 44:357-365). This strategy is, however, not generally applicable due to inefficiencies in both mutagenesis and incorporation of the substrates.

Thus, there is a need for a more efficient system to prepare novel polyketides by inhibiting competitive production of the natural product.

Disclosure of the Invention

The invention is directed to methods to prepare novel polyketides using modified modular polyketide synthase systems wherein directed modification incapacitates the system from using its natural starting material. Novel polyketides can then be synthesized by overriding the starter module and supplying a variety of suitable diketide substrates.

Thus, in one aspect, the invention is directed to a method to prepare a novel polyketide which method comprises providing a thioester diketide substrate to a modular PKS comprising at least two modules under conditions wherein said substrate is converted by said modular PKS to a product polyketide, wherein said PKS has been modified to prevent its utilization of the native starter unit. In other aspects, the invention is directed to the modified modular PKS which is disarmed with respect to utilization of the native starter substrate supplying the initial two carbon unit, and to suitable cells modified to contain this disarmed PKS. The invention is further directed to recombinant materials for production of the modified PKS and to the novel polyketides produced by this system.

Brief Description of the Drawings

Figure 1 shows a schematic representation of the DEBS modular PKS.

Figures 2A-2C show the products of a modified DEBS construct wherein the ketosynthase (KS) in module 1 is disarmed.

Figure 3 shows the processing of 6-dEB derivatives to erythromycin-D derivatives.

Modes of Carrying Out the Invention

The invention provides modular PKS systems which are disarmed with respect to loading the native starting material and their corresponding genes. In a particularly preferred embodiment, the ketosynthase (KS) of module 1 is inactivated so as to prevent competition from the native starter unit. Other approaches to similarly disarming the PKS involve inactivating the acyl transferase (AT) or acyl carrier protein (ACP) functions of module 1.

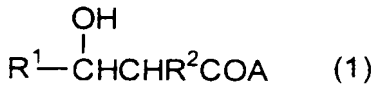
The PKS of the invention must contain at least two modules but may contain additional modules and, indeed may, represent complete synthase systems. While the DEBS PKS system is used to illustrate the invention, any modular PKS can be used, such as the modular PKS resulting in the production of avermectin, rapamycin and the like. Suitable mutations can be introduced by known site specific mutagenesis techniques.

Other micro-organisms such as yeast and bacteria may also be used. When host cells, such as bacteria, yeast, or even mammalian or insect cells, which normally do not produce polyketides are employed, it may be necessary to modify the hosts so as to provide posttranslational processing of the PKS enzymes. Specifically, in order to be functional, the ACP activities must be phosphopantetheinylated. This conversion of an apo-ACP to its activated form is accomplished by enzymes collectively referred to as holo-ACP synthases or PTTases. Forms of these enzymes which function in the fatty acid synthase pathways do not appear to be effective in providing holo-ACP functionalities in the PKS clusters. Thus, importation of a suitable synthase in a recombinant system when the polyketide synthesis is performed in whole cells other than, for example, streptomyces should be employed. If the synthesis is conducted in a cell-free system, the PKS enzymes utilized must have been synthesized under conditions where the holo-ACP synthase is present.

The novel polyketides may thus be synthesized in a suitable hosts, such as a Streptomyces host, especially a Streptomyces host modified so as to delete its own PKS, or other cells modified to produce a suitable PTTase if needed. The polyketides may also be synthesized using a cell-free system by producing the relevant PKS proteins recombinantly and effecting their secretion or lysing the cells containing them. A typical cell-free system would include the appropriate functional PKS, NADPH and an appropriate buffer and substrates required for the catalytic synthesis of polyketides. To produce the novel polyketides thioesters of the extender units are employed along with the thioester of a diketide.

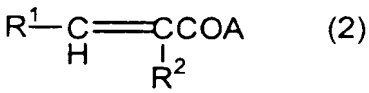
The novel polyketides produced as a result of the modified PKS clusters will differ in the substituents that correspond to the residue of the starter unit in the finished polyketide. And, since the diketide intermediate is being supplied to the

modified PKS cluster, the nature of the extender unit incorporated immediately adjacent the starter unit may also be varied. Thus, the diketides used to make the novel polyketides of the invention are of the general formulas



(1)

or



(2)

wherein A is a moiety that activates the diketide, typically a sulfhydryl such as the N-acetyl cysteamine thioester illustrated below, and at least one of R^1 and R^2 is a substituent other than that natively occurring in the diketide normally processed by the modified PKS cluster. In general, R^1 is a substituted or unsubstituted, saturated or unsaturated hydrocarbyl moiety (1-15C), said hydrocarbyl optionally containing one or two heteroatoms especially N, O or S and R^2 is a substituted or unsubstituted saturated or unsaturated hydrocarbyl moiety (1-4C) or is OR, SR, or NHR, wherein R is substituted or unsubstituted, saturated or unsaturated hydrocarbyl of 1-4C. However, both R^1 and R^2 cannot be methyl and if R^2 is methyl, R^1 cannot be ethyl.

Typical substituents include halo, OR^3, SR^3, NR_2, $-OOCR^3$, $-NHOCR^3$, R^3CO-, R^3COO- and R^3CONH- wherein each R^3 is independently H or lower alkyl (4-4C).

The invention is also directed to polyketides which result from incorporating the diketides of formulas (1) or (2) and to glycosylated forms thereof.

The following examples are intended to illustrate but not to limit the invention.

Preparation A

Starting Materials

Streptomyces coelicolor CH999, which has been engineered to remove the native PKS gene cluster is constructed as described in WO 95/08548. pRM5, a shuttle plasmid used for expressing PKS genes in CH999 was also described in that

application. Plasmid pCK7 which contains the entire DEBS modular system was described in the foregoing application as well.

Example 1

Preparation of DEBS 1+ 2+TE

A modified DEBS PKS system containing only modules 1 and 2 and thioesterase (TE) activity, designated DEBS 1+2+TE, was subjected to site directed mutagenesis to inactivate module 1 KS by replacing the active site cysteine residue in the signature sequence cys-ser-ser-ser-leu by alanine. The resulting expression plasmid, designated pKAO179, encodes a 2-module PKS which is inactive under the standard reaction conditions for synthesis of the native product, *i.e.*, propionyl-CoA, methylmalonyl-CoA, and NADPH. The details of this construction are set forth in Kao, C.M. *et al*, Biochemistry (1996) 35:12363-12368. When provided with the diketide thioester (2S, 3R)-2-methyl-3-hydroxy-pentanoyl-N-acetylcysteamine thioester, and with methylmalonyl-CoA, and NADPH, the triketide product set forth below is obtained.

The triketide product is produced under these conditions when the PKS is incubated in a cell-free system or can be duplicated *in vivo* by providing the appropriate diketide thioester analogs to actively growing cultures of CH99 containing the modified expression plasmid:

A culture of *S. coelicolor* CH999/pKAO179 is established by inoculation of 200 mL of SMM medium (5% PEG-800, 0.06% $MgSO_4$, 0.2% $(NH_4)_2SO_4$, 25 mM TES, pH 7.02, 25 mM KH_2PO_4, 1.6% glucose, 0.5% casamino acids, trace elements) with spores. The culture is incubated at 30°C with shaking at 325 rpm. A solution of (2S, 3R)-2-methyl-3-hydroxypentanoyl N-acetylcysteamine thioester (100 mg) and 4-pentynoic (15 mg) in 1 mL of methylsulfoxide is added to the culture in three parts: after 50 hours (400 mL); after 62 hours (300 mL); and after 86 hours (300 mL). After a total of 144 hours, the culture is centrifuged to remove mycelia. The fermentation broth is saturated with NaCl and extracted with ethyl acetate (5 x 100 mL). The combined organic extract is dried over Na_2SO_4, filtered, and concentrated. Silica gel

chromatography yields (2R, 3S, 4S, 5R)-2,4-dimethyl-3, 5-dihydroxy-n-heptanoic acid δ-lactone.

Example 2

Preparation of Polyketides from the DEBS Cluster

The active site mutated module 1 KS domain of the *eryAI* (DEBS 1 gene) is provided on plasmid pCK7, which contains the *eryAI*, *eryAII* (DEBS 2) and *eryAIII* (DEBS 3 genes) under control of the *actI* promoter. Expression from this plasmid pJRJ2 results in a suitably modified full length PKS system. (Kao, C.M *et al.*, *Science* (1994) 265:409-512. pJRJ2 was transformed into CH999 and grown on R2YE medium. No detectable 6 DEB-like products were produced.

In more detail, lawns of CH999/pJRJ2 were grown at 30°C on R2YE agar plates containing 0.3 mg/ml sodium propionate. After three days, each agar plate was overlayed with 1.5 mL of a 20 mM substrate solution in 9:1 water:DMSO. After an additional 4 days, the agar media (300 mL) were homogenized and extracted three times with ethyl acetate. The solvent was dried over magnesium sulfate and concentrated. Concentrated extracts were purified by silica gel chromatography (gradient of ethyl acetate in hexanes) to afford products.

However, when substrate 2, prepared by the method of Cane *et al.*, *J. Am. Chem. Soc.* (1993) 115:522-526; Cane, D.E. *et al.*, *J. Antibiot.* (1995) 48:647-651, shown in Figure 2 (the NAC thioester of the native diketide) was added to the system, the normal product, 6 dEB was produced in large quantities. Administration of 100 mg substrate 2 to small scale cultures (300 ml grown on petri plates as described above, resulted in production of 30 mg 6 dEB, 18% yield.

Example 3

Production of Novel Polyketides

Diketides with the structures shown in Figure 2A as formulas 3, 4, and 5 were then administered to growing cultures of CH999/pJRJ2 under the conditions of Example 2. Substrates 3 and 4 were prepared as described for Substrate 2 but substituting valeraldehyde and phenylacetaldehyde, respectively for propionaldehyde

in the aldol reactions. The preparation of Substrate 5 was described by Yue, S. *et al.*, *J. Am. Chem. Soc.* (1987) 109:1253-1255. Substrates 3 and 4 provided 55 mg/L of product 6 and 22 mg/L of product 7. respectively. Substrate 5 resulted in the production of 25 mg/L of the 16 member lactone 8, an unexpected product.

Example 4

Additional Novel Polyketides

Diketides with the structures shown in Figures 2B and 2C as compounds 9-18 were administered to growing cultures of CH999/pJRJ2 under the conditions of Example 2. The products were those set forth in Figures 2B and 2C as compounds 19-28.

Example 5

Steric Requirements

Using the same system set forth in Example 2, but substituting for compound 2 the three diasteriomeric forms of the structure of formula 2 shown in Figure 2A, synthesis of a polyketide in each case was not detected. Similarly, substituting for compound 12 its enantiomer at the 2-position, no synthesis of polyketide was detected.

Example 6

Processing of the Polyketide Products

The successful processing of unnatural intermediates by the "downstream" modules of DEBS prompted an experiment to determine whether the post-PKS enzymes in the erythromycin biosynthetic pathway might also accept unnatural substrates. In the natural producer organism, *Saccharopolyspora erythrea*, 6dEB undergoes several enzyme-catalyzed transformations. Oxidation at C6 and glycosylations at C3 and C5 afford erythromycin D (formula 9 in Figure 3) and subsequent transformations afford erythromycins A, B, and C. *S. erythrea* mutant (A34) (Weber, J.M. *et al.*, *J. Bactiol.* (1985) 164:425-433) is unable to synthesize 6dEB. This strain produces no erythromycin when grown on R2YE plates (as judged

by the ability of extracts to inhibit growth of the erythromycin-sensitive bacterium *Bacillus cereus*). However, when 6dEB (which has no antibacterial activity) is added to the culture medium, extracts exhibited potent antibacterial activity.

Samples of 6dEB derivatives 6 and 7 were assayed for conversion by this strain. Partially purified extracts demonstrated inhibition of *B. cereus* growth, and mass spectrometry was used to identify the major components of the extracts as formula 10 in Figure 3 (from 6) and formula 11 (from 7).

In more detail, purified 6 and 7 (5 mg dissolved in 7.5 mL 50% aqueous ethanol) were layered onto R2YE plates (200 mL media/experiment) and allowed to dry. *S. erythrea A34* was then applied so as to give lawns. After 7 days of growth, the media were homogenized and extracted three times with 98.5:1.5 ethyl acetate:triethylamine. Pooled extracts from each experiment were dried over magnesium sulfate and concentrated. Extracts were partially purified by silica gel chromatography (gradient of methanol and triethylamine in chloroform). The partially purified extracts were examined by TLC and mass spectrometry. For antibacterial activity analysis, filter discs were soaked in 400 μM ethanolic solutions of erythromycin D, 10 and 11, as well as a concentrated extract from *S. erythrea A34* which had been grown without addition of any 6-dEB analogs. Disks were dried and laid over freshly-plated lawns of *Bacillus cereus*. After incubation for 12h at 37°C, inhibition of bacterial growth was evident for all compounds but not for the control extract.

Claims

1. A modified functional modular polyketide synthase (PKS) comprising at least two modules, wherein said PKS has been modified to prevent its utilization of the native starter unit for said modular PKS, wherein the ketosynthase (KS) catalytic domain of module 1 has been inactivated.

2. The modified PKS of claim 1 which is a complete PKS.

3. A PKS gene cluster which encodes a modified PKS wherein said modified PKS has been modified to prevent its utilization of the native starter unit for said modular PKS, wherein the ketosynthase (KS) catalytic domain of module 1 has been inactivated.

4. The gene cluster of claim 3 which encodes a complete PKS.

5. A recombinant host cell modified to contain the gene cluster of claim 3 which further optionally includes a recombinant expression system for the production of a holo-ACP synthase effective to phosphopantetheinylate the ACP regions of the PKS produced.

6. A method to prepare a polyketide, which method comprises providing a thioester diketide substrate for the modified PKS of claim 1.

7. The method of claim 6 which is conducted in a host cell.

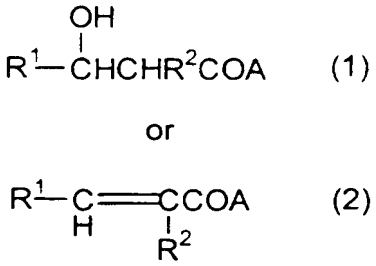
8. The method of claim 6 which is conducted in a cell free system.

9. A method to prepare an antibiotic which method comprises treating the polyketide product of the method of claim 6 with an enzyme that effects polyketide glycosylation.

10. A novel polyketide which has the structure shown as formula 6-8 in Figure 2A or formulas 19-28 of Figures 2B and 2C.

11. A novel antibiotic which is the glycosylated form of the polyketide of claim 10.

12. A novel polyketide which is obtainable by the method of claim 6 from the diketide of the formulas



(1)

or

(2)

wherein A is a moiety that activates the diketide;

at least one of R^1 and R^2 is a substituent other than that natively occurring in the diketide normally processed by the modified PKS cluster; and wherein

R^1 is a substituted or unsubstituted, saturated or unsaturated hydrocarbyl moiety (1-15C), said hydrocarbyl optionally containing one or two heteroatoms and R^2 is a substituted or unsubstituted, saturated or unsaturated hydrocarbyl moiety (1-4C) or is OR, SR, or NHR, wherein R is substituted or unsubstituted, saturated or unsaturated hydrocarbyl of 1-4C with the proviso that both R^1 and R^2 cannot be methyl and if R^2 is methyl, R^1 cannot be ethyl.

13. A novel antibiotic which is the glycosylated form of the novel polyketide of claim 12.

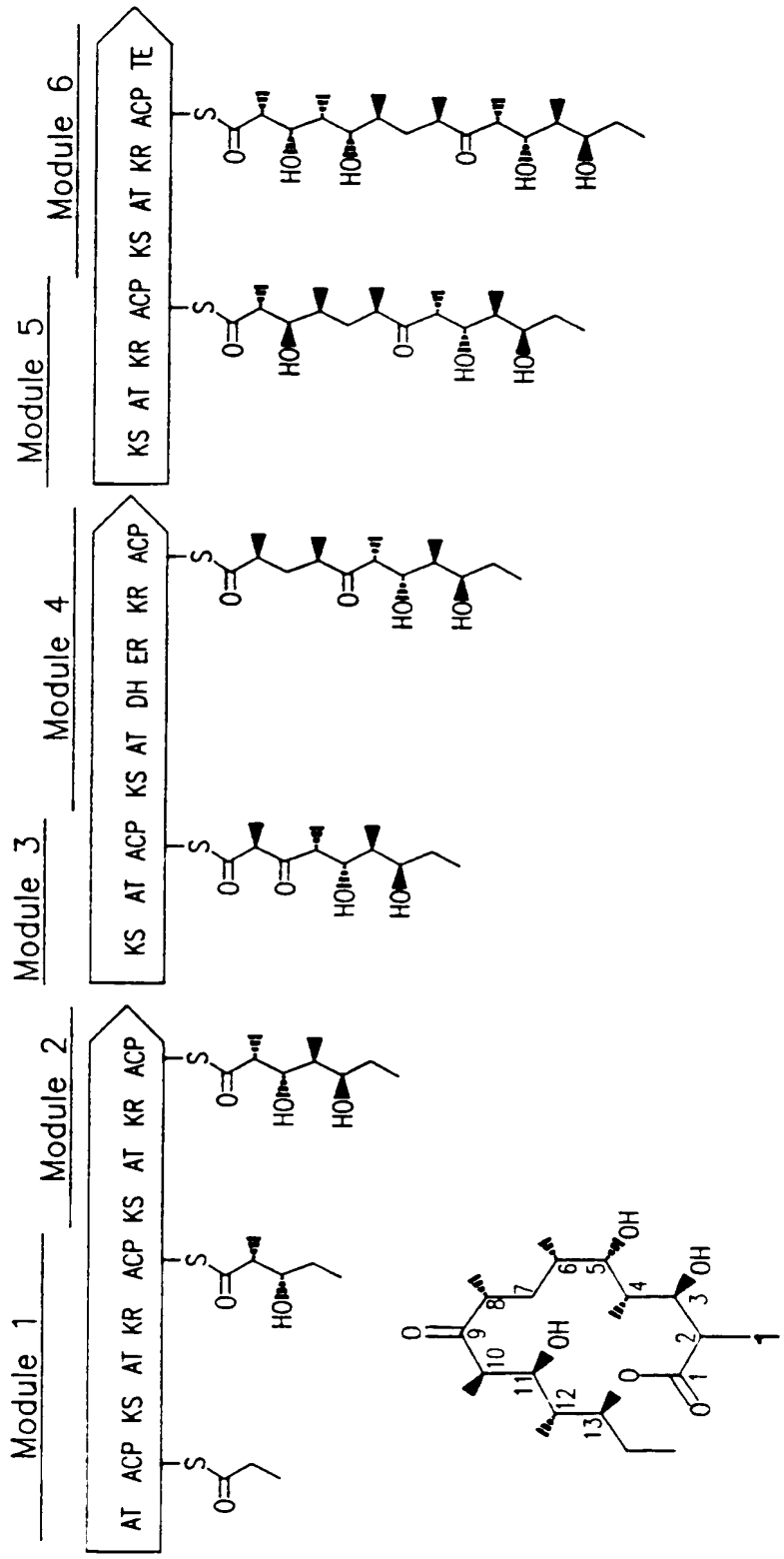


FIG. 1

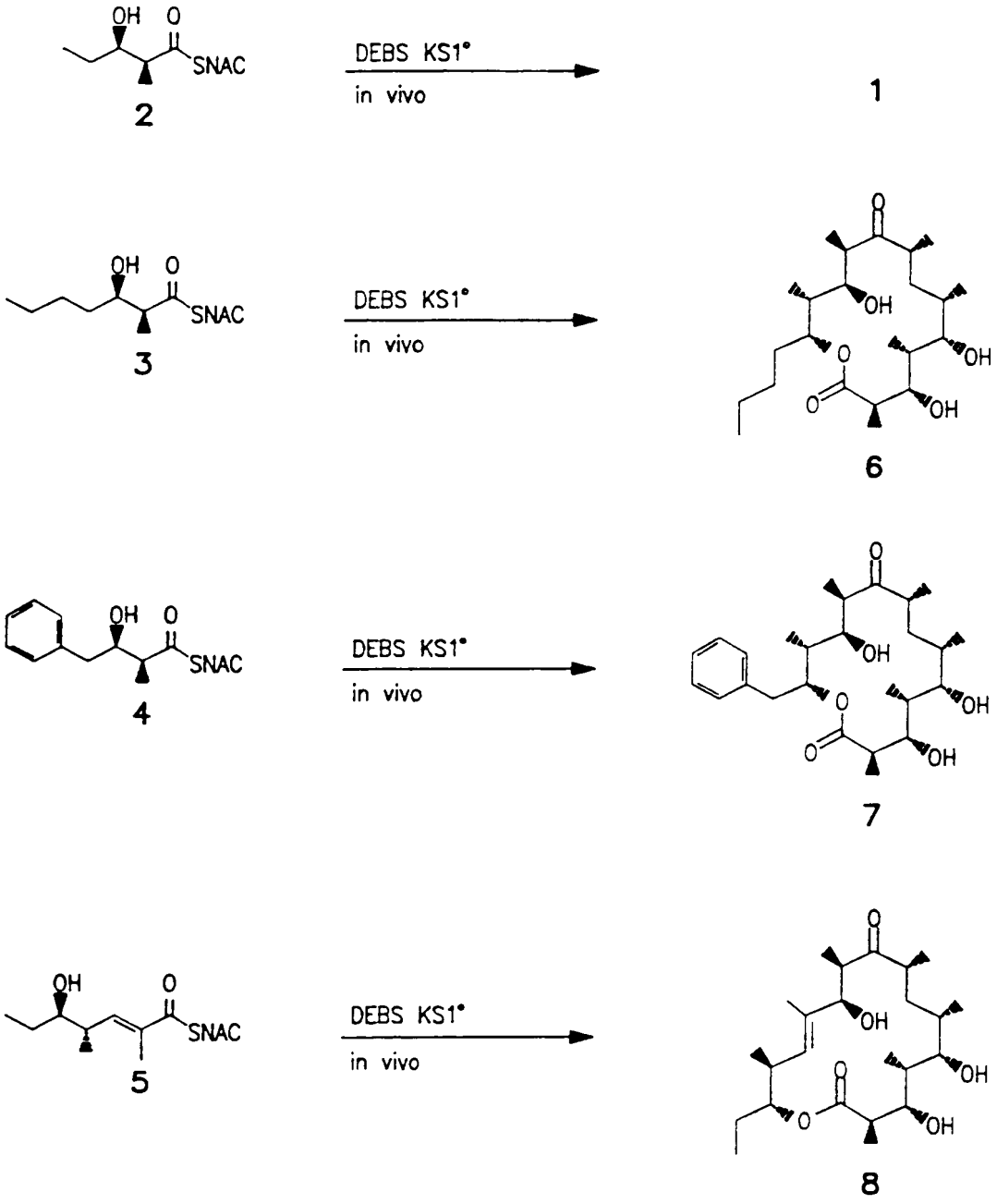


FIG. 2A

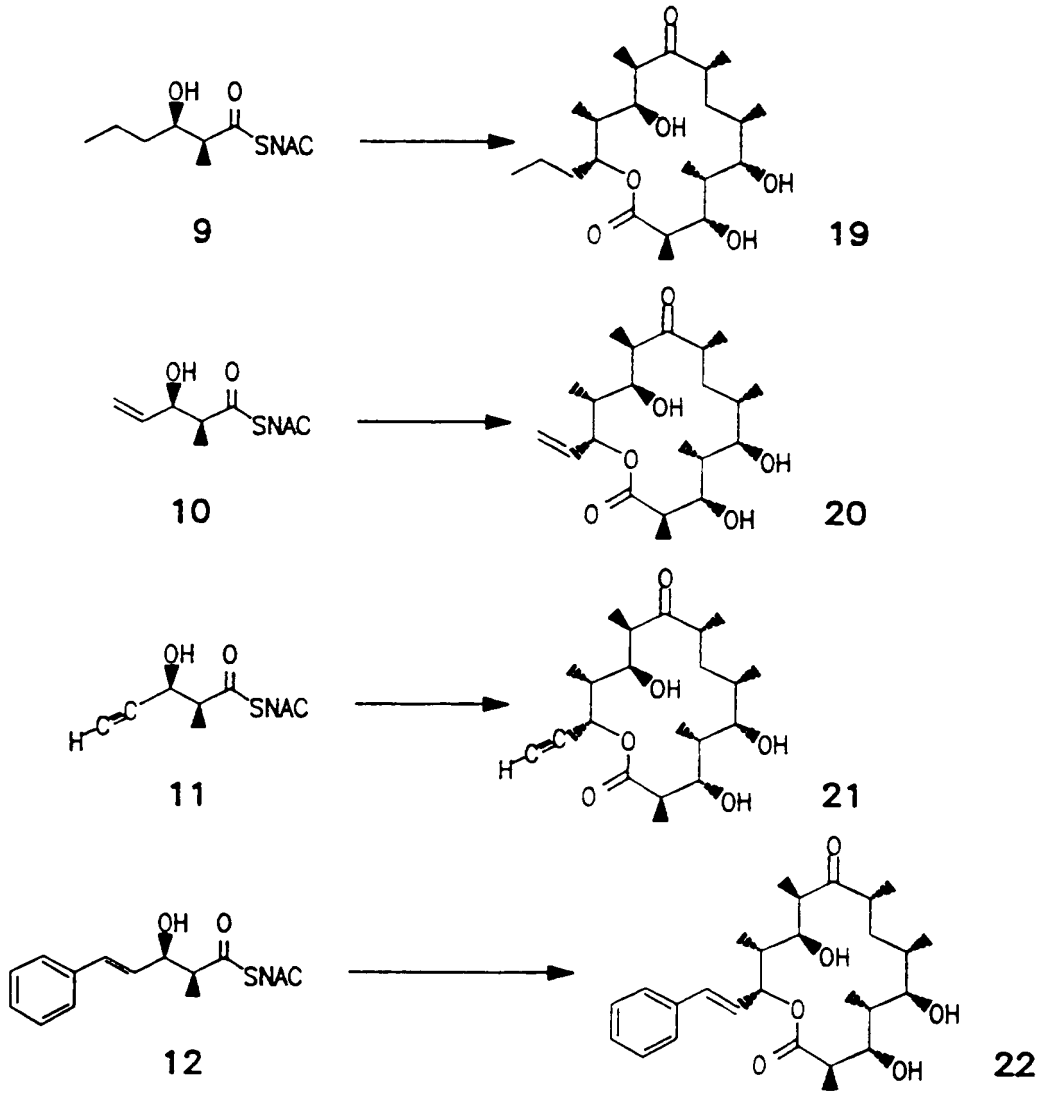


FIG. 2B

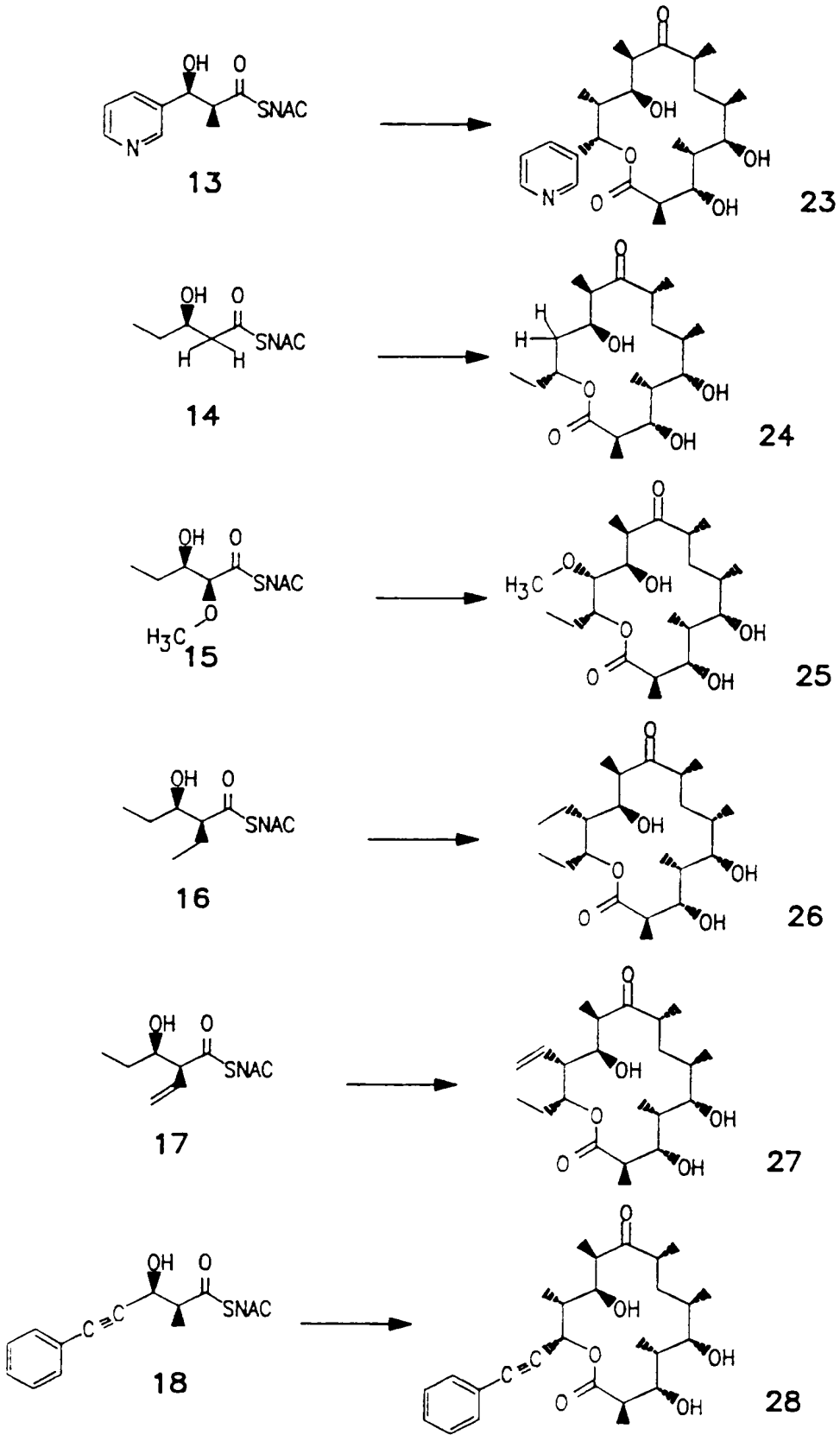
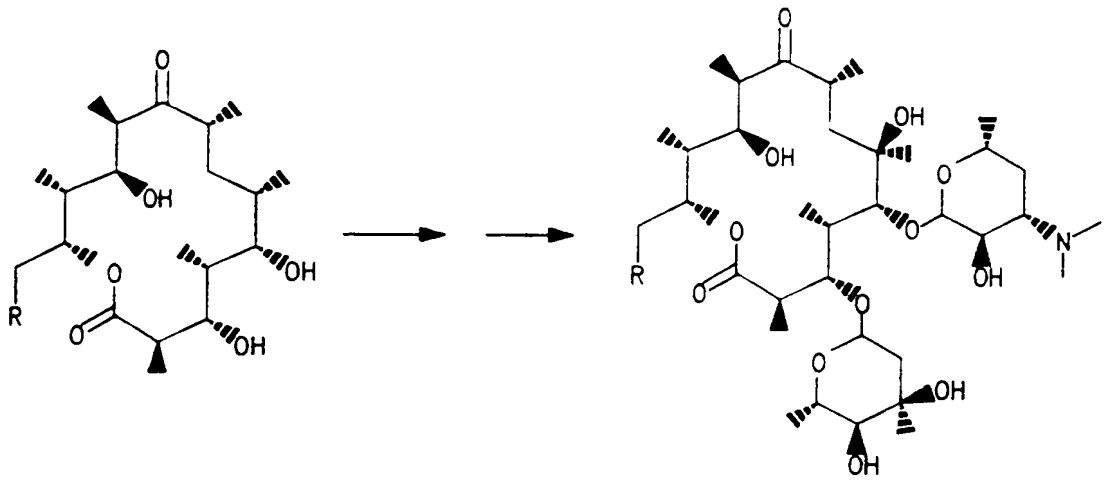


FIG. 2C



1: R=Methyl
6: R=n–Propyl
7: R=Phenyl

9: R=Methyl
10: R=n–Propyl
11: R=Phenyl

FIG. 3

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 15/52, 9/10, C12P 17/08 — A3

(11) International Publication Number: **WO 99/03986**

(43) International Publication Date: 28 January 1999 (28.01.99)

(21) International Application Number: PCT/US98/14911

(22) International Filing Date: 17 July 1998 (17.07.98)

(30) Priority Data:
08/896,323 17 July 1997 (17.07.97) US

(71) Applicant: KOSAN BIOSCIENCES, INC. [US/US]; 1450 Rollins Road, Burlingame, CA 94010 (US).

(72) Inventors: KHOSLA, Chaitan; 132 Peter Courts Circle, Stanford, CT 94305 (US). PIEPER, Rembert; 957 Menlo Avenue, Menlo Park, CA 94025 (US). LUO, Guanglin; Apartment #3, 80 Preston Street, Providence, RI 02906 (US). CANE, David, E.; 35 Laural Avenue, Providence, RI 02906 (US).

(74) Agents: MURASHIGE, Kate, H. et al.; Morrison & Foerster LLP, 2000 Pennsylvania Avenue, N.W., Washington, DC 20006-1888 (US).

(81) Designated States: AL, AM, AU, AZ, BA, BB, BG, BR, CA, CN, CU, CZ, EE, FI, GE, HU, IL, IS, JP, KG, KP, KR, LC, LK, LR, LT, LV, MD, MG, MK, MN, MX, NO, NZ, PL, RO, SG, SI, SK, TR, TT, UA, UZ, VN, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

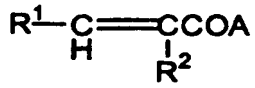
(88) Date of publication of the international search report:
8 April 1999 (08.04.99)

(54) Title: METHOD TO PRODUCE POLYKETIDES AND POLYKETIDES

(57) Abstract

Modified PKS gene clusters which produce novel polyketides in an efficient system in a host cell or in a cell free extract are described. The novel polyketides result from the incorporation of diketides of formula (1) or (2), wherein A is a moiety that activates the diketide, and at least one of R^1 and R^2 is a substituent other than that natively occuring in the diketide normally processed by the modified PKS cluster. The polyketides may also be glycosylated to provide antibiotics.

(1)



(2)

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	**ES**	Spain	**LS**	Lesotho	**SI**	Slovenia
AM	Armenia	**FI**	Finland	**LT**	Lithuania	**SK**	Slovakia
AT	Austria	**FR**	France	**LU**	Luxembourg	**SN**	Senegal
AU	Australia	**GA**	Gabon	**LV**	Latvia	**SZ**	Swaziland
AZ	Azerbaijan	**GB**	United Kingdom	**MC**	Monaco	**TD**	Chad
BA	Bosnia and Herzegovina	**GE**	Georgia	**MD**	Republic of Moldova	**TG**	Togo
BB	Barbados	**GH**	Ghana	**MG**	Madagascar	**TJ**	Tajikistan
BE	Belgium	**GN**	Guinea	**MK**	The former Yugoslav Republic of Macedonia	**TM**	Turkmenistan
BF	Burkina Faso	**GR**	Greece			**TR**	Turkey
BG	Bulgaria	**HU**	Hungary	**ML**	Mali	**TT**	Trinidad and Tobago
BJ	Benin	**IE**	Ireland	**MN**	Mongolia	**UA**	Ukraine
BR	Brazil	**IL**	Israel	**MR**	Mauritania	**UG**	Uganda
BY	Belarus	**IS**	Iceland	**MW**	Malawi	**US**	United States of America
CA	Canada	**IT**	Italy	**MX**	Mexico	**UZ**	Uzbekistan
CF	Central African Republic	**JP**	Japan	**NE**	Niger	**VN**	Viet Nam
CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

INTERNATIONAL SEARCH REPORT

Intern nal Application No
PCT/US 98/14911

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/52 C12N9/10 C12P17/08

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KAO CM ET AL: "Evidence for two catalytically independent clusters of active sites in a functional modular polyketide synthase." BIOCHEMISTRY, SEP 24 1996, 35 (38) P12363-8, XP002082318 UNITED STATES	1-7,9
Y	see the whole document	8

Y	WO 97 02358 A (UNIV LELAND STANFORD JUNIOR ;UNIV BROWN RES FOUND (US)) 23 January 1997 see claims 1-16; figure 1	8

A	WO 97 13845 A (UNIV CALIFORNIA SAN FRANCISCO ;HARVARD COLLEGE (US)) 17 April 1997 see abstract; claims 28-30	5

	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
27 October 1998	08.02.99

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax. (+31-70) 340-3016	Gurdjian, D

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Interr ·nal Application No
PCT/US 98/14911

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DUTTON CJ ET AL: "Novel avermectins produced by mutational biosynthesis" JOURNAL OF ANTIBIOTICS, vol. 44, no. 3, 1991, pages 357-365, XP002079430 cited in the application see the whole document	1-9

1

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 98/14911

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4 ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-9

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

1. Claims: 1-9

modified functional modular polyketide synthase comprising at least two modules , wherein the ketosynthase of the first module has been inactivated , corresponding gene cluster , recombinant host cells , method to prepare a polyketide

2. Claims: 10-13

Polyketides with formulas 6-8, 19-28 or obtained with precursors thioester diketide substrate with formula (1) or (2) of claim 12

INTERNATIONAL SEARCH REPORT

ormation on patent family members

Inter onal Application No
PCT/US 98/14911

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9702358 A	23-01-1997	AU 6542696 A	05-02-1997
		CA 2226221 A	23-01-1997
		EP 0836649 A	22-04-1998
		JP 10510167 T	06-10-1998
WO 9713845 A	17-04-1997	AU 7435796 A	30-04-1997
		EP 0861321 A	02-09-1998

Form PCT/ISA/210 (patent family annex) (July 1992)